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A MORPHOLOGICAL EXAMINATION OF SPERM-EGG INTERACTION IN THE FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

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ABSTRACT

Eggs, covered by a bilayered investment composed of a 0.5 μm protein outer layer and a 2.5 μm mucopolysaccharide inner layer, are spawned through an externally held spermatophore following a female's ovigerous molt and mating. Mature sperm resemble everted umbrellas and consist of a cupped base with a single spike projecting from the convex surface. These sperm (<5/egg) attach base-first with the spike oriented perpendicularly to the investment. Within 15 seconds, the spike of the sperm bends at the base and contacts the investment. The spike penetrates the investment, the base is inverted, and fertilization occurs within two minutes. The spike remains briefly as a central core in the fertilization cone. The meiotic division of the egg resumes at this stage. First karyokinesis is completed approximately 6 hours post fertilization, but cytokinesis is suppressed until following the second karyokinesis at 8 hours postfertilization. First and second cytokinesis are simultaneous, and at 9 h the egg cleaves to the four cell stage.

INTRODUCTION

The sequence of morphological events leading ultimately to gamete fusion have been well documented in several vertebrate and invertebrate species (Austin, 1968; Epel and Vacquier, 1978, for review). In most of the gamete systems studied, motile male gametes are involved and follow the classic sea urchin fertilization model with minor modifications. Sperm approach the egg head first and encounter one or more investments. An acrosome reaction may occur at this time, allowing the sperm to recognize and bind to the vitelline layer. Cell fusion then occurs between the inner acrosomal membrane of the reacted sperm and the egg plasma membrane. This point of gamete fusion is often characterized by a fertilization cone.

In contrast, fertilization in the animal kingdom involving non-motile male gametes is poorly understood, particularly in the decapod crustaceans. Sperm of the decapods are considered atypical, nonmotile gametes and are separated into the multistellate sperm of the reptantians (crabs, crayfish, lobsters) and the unistellate sperm of the natantians (shrimp) (Wilson, 1928; Lu, 1976; Talbot and Summers, 1978). Although fertilization has been studied in both groups, the events that occur during reptantian fertilization are more extensively documented.

Early workers often reported observations of "exploded" or "everted" sperm in the reptantians (*e.g.*, Labbe, 1904; Koltzoff, 1906; Retzius, 1909; Binford, 1913; Fasten, 1924). Only a few of these early workers attempted to relate the sperm eversion process to a necessary step in fertilization (Koltzoff, 1906; Binford, 1913)

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rather than a result of a simple change in osmotic pressure as suggested by other authors (Labbe, 1904; Koltzoff, 1906; Retzius, 1909). Subsequent investigators utilizing electron microscopy demonstrated that the explosive eversion of the sperm was involved in the penetration of the egg chorion (Brown, 1966; Hinsch, 1971; Goudeau, 1982). Sperm initially contact the egg with their stellate arms and bring the apical cap of the central cup of the sperm containing the acrosome and nucleus into direct contact with the egg investment. The sperm then "explosively everts" and penetrates the chorion to make contact with the oolemma. Both Hinsch (1971) and Brown (1966) have speculated that penetration of the egg chorion is at least partially facilitated by the release of lytic enzymes from the sperm. Actual membrane fusion between the gametes has not been observed.

Fertilization in natantians was erroneously described using light microscopy in a marine shrimp, *Penaeus japonicus* (Hudinaga, 1942). More recently sperm-egg orientation has been described at both the light and electron microscopy level in the shrimp *Sicyonia ingentis* (Clark, *et al.,* 1981a). These authors document an initial "spike first" orientation necessary for egg recognition and essential for the subsequent acrosome reaction to be effective in initiating sperm-egg fusion. As the acrosome reaction starts, the spike apparently depolymerizes, drawing the sperm into close association with the egg surface (Clark *et al.,* 1981a). The acrosome reaction proceeds (Yudin *et al.,* 1979; Clark *et al.,* 1981a), with resultant gamete binding (Yudin *et al.,* 1980; Clark *et al.,* 1981a). Similar observations on sperm-egg orientation and gamete activation have been noted in *Penaeus setiferus* and *P. aztecus* (Clark *et al.,* 1980). This sperm-egg orientation appears to be typical of the family Penaeidae (broadcast spawners).

Although Chow *et al.* (1982) briefly report sperm penetration of the egg of *Macrobrachium rosenbergii,* a complete description of sperm-egg interaction is lacking in the family Palaemonidae (shrimp which brood). Sperm of the palaemonid shrimp are often described as a "thumb tack" or "everted umbrella" shaped cell and consist of a cup-shaped main body with a single appendage (spike) (Nath, 1937; Brown, 1967; Pochon-Masson, 1968; Koehler, 1979; Clark *et al.,* 1980; Sandifer and Lynn, 1981; Chow *et al.,* 1982). An acrosomal region has not been clearly defined in the palaemonid sperm, although Koehler (1979) speculates a perinuclear veiscular region may function in an acrosomal capacity. In contrast, Pochon-Mason (1968, 1969) refers to the single spike as the acrosome and percutor organ. An explosive acrosome reaction as in the reptantians (Brown, 1966; Talbot and Summers, 1978) or elaborate acrosome reaction as in the penaeid shrimp, *S. igentis,*(Yudin *et al.,* 1979; Clark *et al.,* 1981a) is not inducible or observed in the sperm of the palaemonid shrimp (Lynn, unpublished).

Sandifer and Lynn (1981) have reported that the mature egg of *M. rosenbergii* is surrounded by an investment layer 3–5 μm thick and can be divided into two histochemically distinct regions; a proteinaceous, thin outer layer and a PAS positive inner layer. Orientation of the sperm to the egg and subsequent events leading to penetration of this formidable egg investment have thus far only been speculated on (Brown, 1967; Koehler, 1979; Sandifer and Lynn, 1981; Chow *et al.,* 1982). In the present paper the early events of sperm-egg interaction leading ultimately to gamete fusion are examined.

Materials and Methods

Male and female *M. rosenbergii* were obtained from the Institute of Marine Resources, Charleston, South Carolina. These animals are the Anuenue strain orig-

inally brought from Hawaii. Animals were held individually in ten gallon aquaria at 25°C. Females which had undergone an ovigerous molt were mated immediately with a male and checked after several hours for the presence of a spermatophore (Sandifer and Lynn, 1981). When females had successfully mated and spermatophores were present, the males were removed from the aquarium and the females were observed continuously until spawning.

Collection of spawned, fertilized eggs was facilitated by removal of the endopodites and exopodites of the second pair of pleopods allowing the fertilized eggs to fall to the aquarium bottom. Eggs were sampled at 15 second intervals post spawning with a large bore pipet for the first fifteen minutes after the initiation of spawning. For light microscopy, samples were fixed 8–10 hours in 4% glutaraldehyde with 3% sucrose in a 0.1 *M* PO_4 buffer at pH 7.4. The eggs were postfixed one hour in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in a low viscosity epoxy resin (Spurr, 1969). Thick sections were cut with glass knives on a Sorvall MT-2B ultramicrotome and stained with borate buffered 0.5% toluidine blue at pH 11.0.

For scanning electron microscopy, samples were collected and fixed as above, dehydrated in a graded acetone series, critically point dried in CO_2, and scattered on SEM studs. Eggs were then sputter coated with 25 nm gold in a Polaron sputter coater and observed in a Philips SEM 501 scanning electron microscope at an accelerating voltage of 10–15 kV.

Results

Fertilization in *M. rosenbergii* is external, occurring as the eggs are released from the gonopores at the base of the third pair of pereiopods and pass posteriorly over the spermatophore mass. The pleopods and thoracic coxae are positioned to form an enclosed channel for the eggs which ensures egg contact with the spermatophore mass.

Mature eggs removed from the ovary of an ovigerous female are approximately 500 μm in diameter and densely packed with yolk (Fig. 1). A germinal vesicle is not present and a single 3–5 μm investment surrounds the egg at this time. The investment layer may be resolved into a thin 0.5 μm porous outer layer and a much thicker 2.5 μm spongy inner layer (Fig. 2). Structures resembling micropyles are not observed in the thin outer layer when examined in thin plastic sections or with SEM.

Fertilized eggs collected immediately after spawning (0 time) generally have three to five sperm attached (Fig. 3). Sperm are oriented with their bases in contact with the investment and the spike erect (Fig. 4, 5). Numerous filamentous strands apparently originating from the base of the sperm, are intimately associated with the egg investment (Fig. 4). Cross sections of the sperm reveal the marked cupped appearance of the base as it sits on the investment resulting in contact being primarily restricted to the periphery of the sperm base (Fig. 5). During the next two minutes post spawning, a series of events involving changes in sperm orientation and penetration of the investment occurs and terminates with the incorporation of the sperm into the egg.

Fifteen to 30 seconds post spawning, the spike of the sperm begins to bend until the tip makes contact with the egg investment (Figs. 6, 7, 8). This bending characteristically occurs at the base of the spike. Cross sections of the sperm show the prominent cup shape of the base has been lost and a more intimate contact between the entire sperm base and the investment is achieved (Fig. 7). The filamentous extensions of the sperm base are still closely associated with the egg investment.

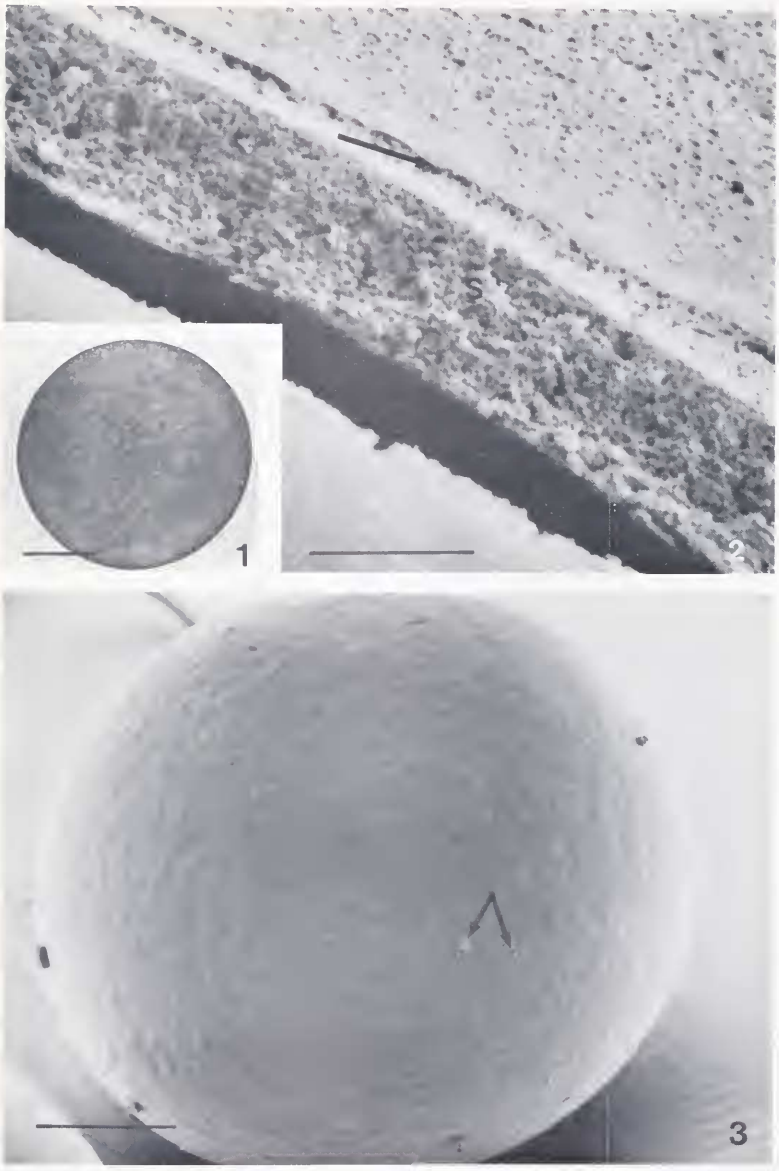

FIGURE 1. A mature *Macrobrachium rosenbergii* egg is approximately 0.5 mm in diameter and has a homogenous cytoplasm when viewed with phase microscopy. Bar = 200 μm.

FIGURE 2. The egg is covered by a bilayered investment and consists of a thin outer layer (arrow) which appears porous in scanning electron microscopy and a much thicker spongy layer (S) which remains closely opposed to the egg before fertilization. Bar = 5 μm.

FIGURE 3. The external surface of the egg investment has no differentiated area such as a micropyle, and following insemination, only 2–5 sperm (arrows) attach to the surface. Bar = 100 μm.

A breach in the outermost layer of the investment around the tip of the spike begins to appear by 45 seconds (Fig. 9). At this time, the base of the sperm partially dissociates from the egg investment and the edge distal to the point of contact lifts

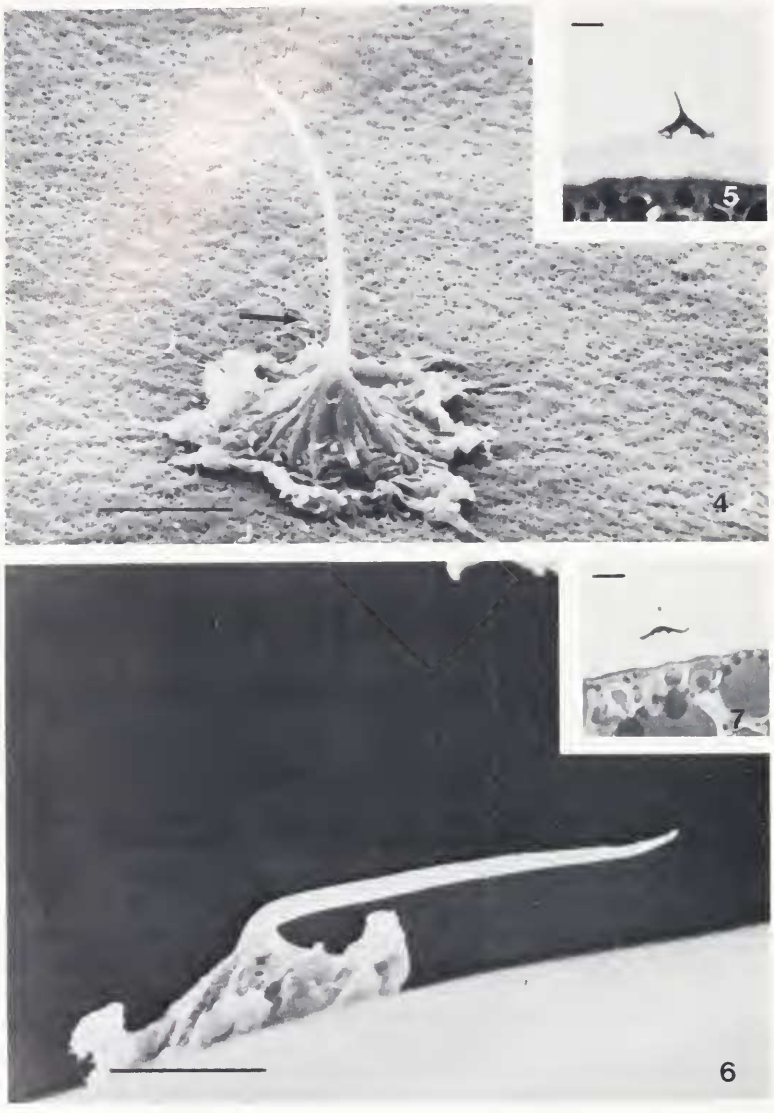

FIGURE 4. Mature sperm attach to the egg surface base first at fifteen seconds postspawning. The spike is diametrically opposed to the egg surface and filamentous strands from the sperm are associated with the egg investment (arrow). Bar = 5 μm.

FIGURE 5. In thick plastic sections stained for light microscopy, the sperm base retains a marked cupped shape. Bar = 5 μm.

FIGURE 6. By fifteen to 30 seconds postspawning, the sperm spike bends close to the base. Bar = 5 μm.

FIGURE 7. In cross sections, the sperm base has noticeably flattened during the spike bending. Bar = 5 μm.

off of the investment surface (Figs. 9, 10). Filamentous extensions are no longer apparent in the disassociated region of the base (Fig. 10). Any evidence of former attachment in terms of investment scarring is not apparent.

By the end of 60 seconds, the spike is entering a breach in the investment (Fig. 11). At this time, the base of the sperm is almost entirely dissociated from the

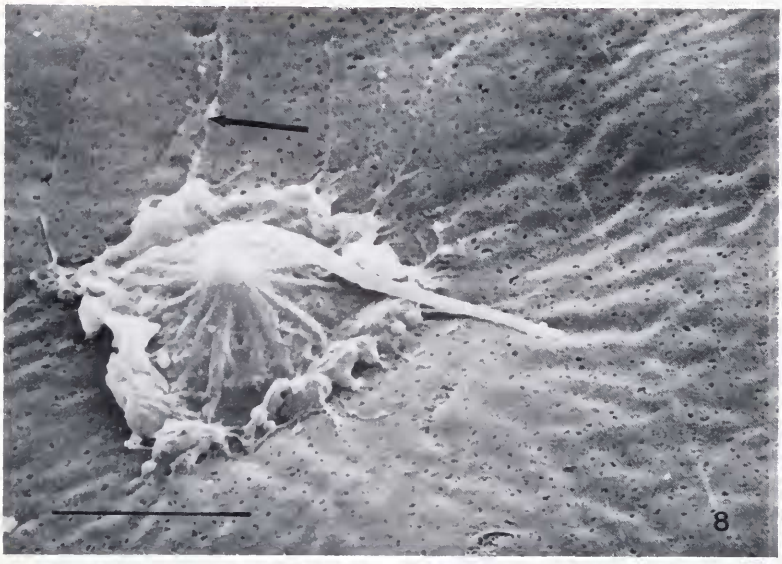

FIGURE 8. At the end of 30 seconds following spawning, the spike has contacted the egg investment. The base remains flattened, and filamentous material is still associated with the egg surface (arrow). Bar = 5 μm.

FIGURE 9. A breach in the investment surface begins to appear around the tip of the spike (arrow) by 45 seconds and the base of the sperm distal to the point of spike egg contact is detached from the egg surface. Bar = 1μm.

investment and in subsequent stages is entirely lifted off (Fig. 12). Some residual filamentous material may still link the base with the investment (Fig. 11). As noted earlier, no scarring of the investment is discernible where the base had originally contacted the investment.

Fusion has occurred by 90 seconds post spawning (Fig. 13, 14) and a fertilization cone is apparent. A dense core, perhaps representing elements of the spike, can be distinguished in the cone and is surrounded by densely staining cytoplasm (Fig. 14).

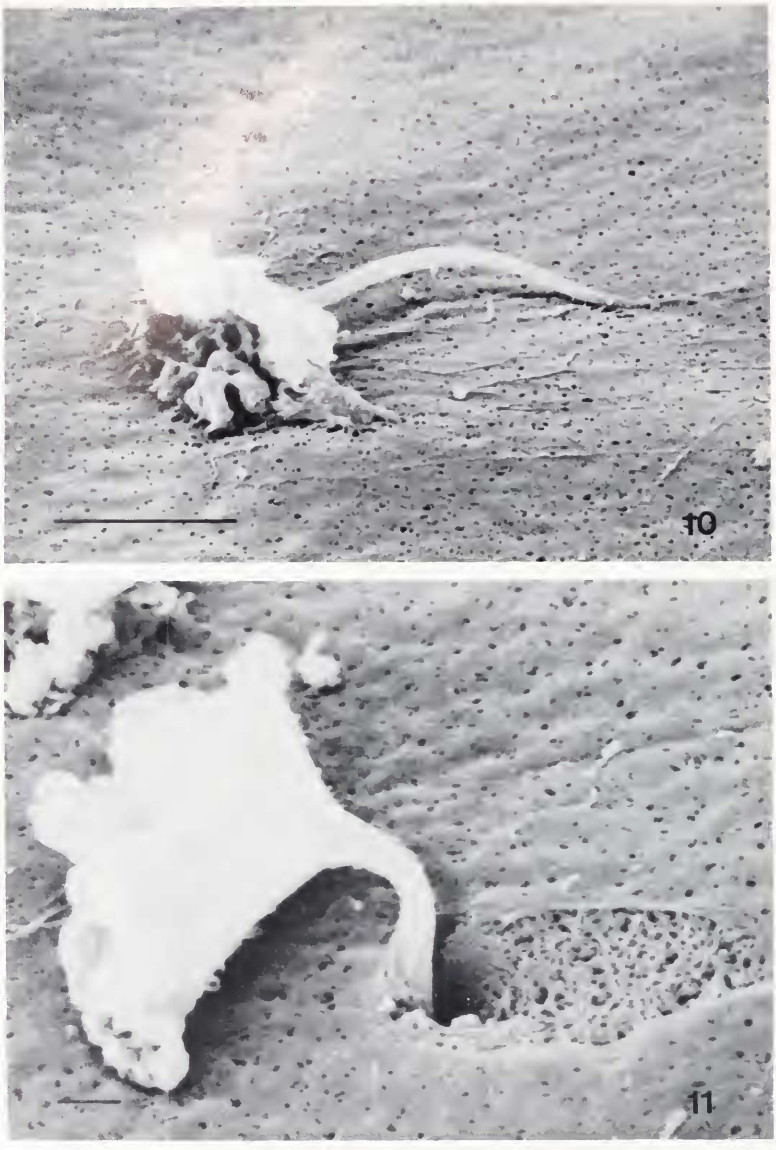

FIGURE 10. Filamentous material is now only associated with the egg at the point of contact of the sperm base. No scarring in the vicinity of the original attachment of the sperm base is visible. Bar = 5 μm.

FIGURE 11. Penetration of the spike through the egg investment continues and the breach in the investment surface enlarges. The cupped shape of the sperm base has returned by 60 seconds. Bar = 1 μm.

At this stage, the sperm base, now inverted from its original orientation to the egg, is in close association with the fertilization cone and lies within a funnel-shaped breach in the investment (Fig. 13, 14). The breach in the investment continues to enlarge, the fertilization cone diminishes in size and only remnants of the sperm base are apparent (Fig. 15, 16). Two minutes post spawning, a male pronucleus is

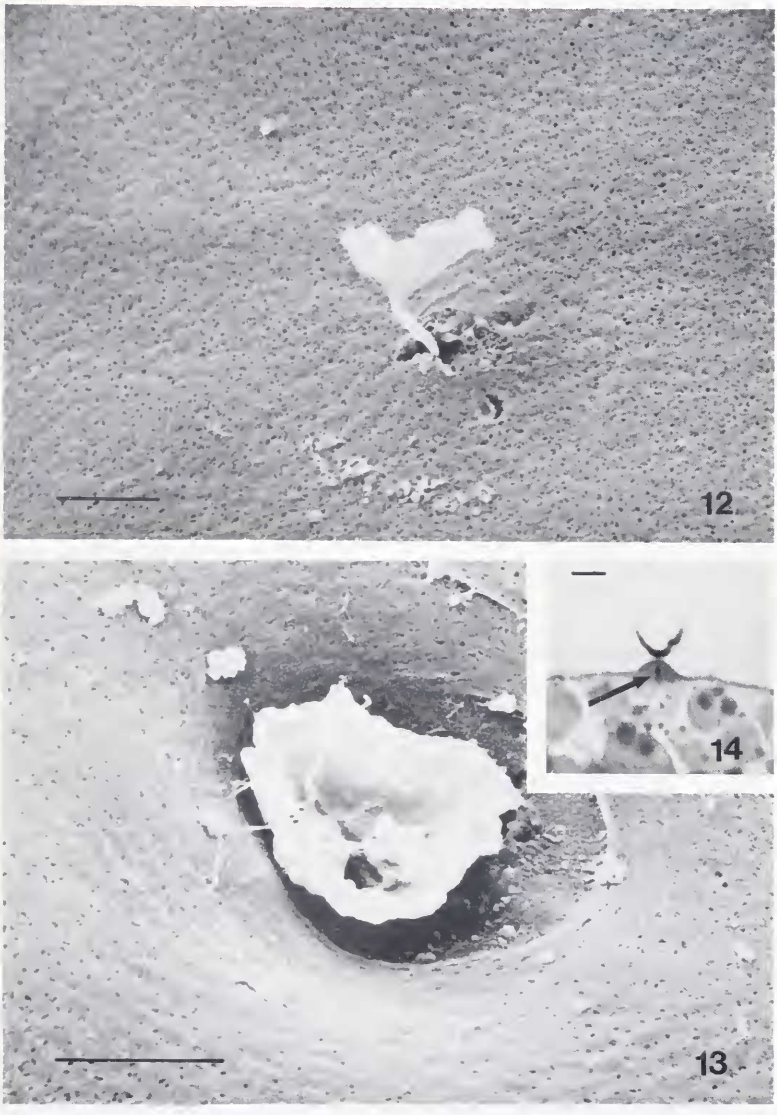

FIGURE 12. Rapidly the sperm base is lifted off the egg surface and is inverted over the spike and the breach in the investment surface. Notice that there is no additional scarring of the investment except in the immediate vicinity of the sperm spike. Filamentous material associated with the sperm base and the investment are now almost completely absent. Bar = 5 μm.

FIGURE 13. By the end of 90 seconds, the base of the sperm rests in a funnel shaped depression in the investment. The breach in the thin outer layer now exceeds the diameter of the sperm base. Bar = 5 μm.

FIGURE 14. At the light level, the formation of a fertilization cone is observed and the spike of the sperm remains briefly as a dense central core (arrow). Note the tapered funnel shape of the investment in the area of the sperm base. Bar = 5 μm.

formed (Fig. 17). Meiotic divisions of the egg resume during the latter stages of sperm fusion, and meiotic figures are often observed at 5 to 10 minutes post fertilization (Fig. 18). Although monospermic fertilization appears to be the rule, in-

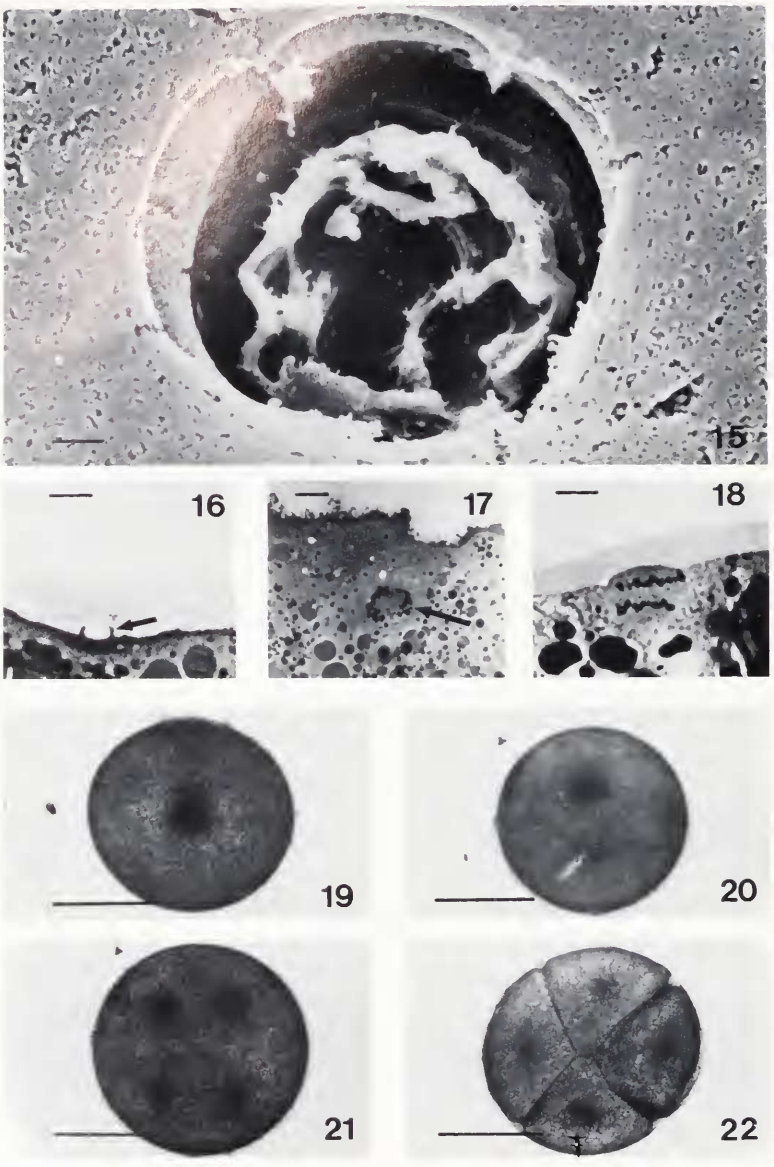

FIGURE 15. Within two to three minutes following spawning, the sperm has been incorporated into the egg and only remnants of the sperm base are visible with scanning electron microscopy. The breach in the investment surface will remain visible for at least twenty minutes post insemination. Bar = 1 μm.

FIGURE 16. The sperm base is often visible as a cup shaped remnant (arrow) at the surface of the plasmalemma at this stage, and the forming sperm pronucleus is visible as a thin dark band immediately beneath the base. Bar = 10 μm.

FIGURE 17. By 3–5 minutes, the point of sperm entry into the egg is identifiable only as a depression in the plasmalemma. The sperm pronucleus (arrow) has now begun to move into the egg cytoplasm. Bar = 10μm.

FIGURE 18. Concurrent with the penetration stage, the egg resumes its meiotic division and meiotic figures such as this one arrested during anaphase are often observed. Bar = 5 μm.

FIGURE 19. Four hours after spawning the zygote nucleus is centered in the egg cytoplasm. Bar = 250 μm.

stances of multispermic penetration were observed. In this study, only one in 500 eggs examined was polyspermic. Evidence of sperm penetration through the investment persists and may be observed up to 20 minutes post spawning.

At 28°C the nucleus initially appears at about 4 hours as a central dark stellate body in the central egg cytoplasm (Fig. 19), and the first mitotic karyokinesis occurs 6 hours post spawning (Fig. 20). Cytokinesis does not occur at this time and 2.5 to 3.0 hours after first karyokinesis, a second nuclear division produces four dense nucleoplasmic regions in the cytoplasm (Fig. 21). Approximately two hours after the second nuclear division (ten hours post spawning) the first and second cleavage divisions occur simultaneously resulting in four equally sized blastomeres (Fig. 22). Subsequent nuclear and cytoplasmic divisions occur in normal sequence.

Discussion

Two unique features characterize the sperm egg interaction observed in *M. rosenbergii*. First, there is a dramatic orientation-reorientation of the sperm following initial sperm-egg contact. Second, sperm of *M. rosenbergii* do not undergo an acrosome eversion as seen in the reptantians (Binford, 1913; Brown, 1966; Hinsch, 1971; Summers, 1978; Chanmanon, 1980), or an obvious acrosome reaction as observed in the natantian group Penaeidea (Yudin *et al.*, 1979; Clark *et al.*, 1981a, b).

In the present paper, it has been demonstrated that initial sperm-egg contact involves a base first orientation of the sperm to the egg investment. Koehler (1979) and Brown (1967) suggested such a base first orientation with the spike diametrically opposed in the caridean shrimp. This configuration seemed logical, since PAS positive vesicles were reported in the perinuclear region of the base by Koehler (1979) who suggested that they might serve as an acrosome. Brown (1967) has suggested a similar configuration based on preliminary observations on attempted *in vitro* fertilizations with the gametes of *Palaemonetes paludosus*. These authors never observed incorporation of the sperm into the egg, however. Chow *et al.* (1982) proposed that the sperm-egg interaction is based on a spike first approach based on light microscopic observations of sperm penetration into the egg. Early sperm egg interaction has not been reported, however in the caridean shrimp until the present paper. A similar reorientation of a sperm, once apparent sperm-egg binding has occurred, has never been reported in any animal system.

It is interesting to note that Yudin *et al.* (1980; see also, Clark *et al.*, 1981a) have demonstrated that successful sperm-egg contact in the Penaeidae is dependent on a spike-first orientation and is essential in *S. ingentis* for recognition binding to occur. In the case of the Penaeidea shrimp, it has been proposed that the depolymerization of the spike draws the sperm into more intimate contact with the egg (Kleve *et al.*, 1980; Yudin *et al.*, 1980; Clark *et al.*, 1981a) prior to the final stages of sperm-egg fusion 30 to 40 minutes later (Clark *et al.*, 1981a).

Sperm-egg binding after initial contact has been studied in several invertebrate groups (Vacquier and Moy, 1977; Brandiff *et al.*, 1978; Yudin *et al.*, 1980; Clark *et al.*, 1981a). In shrimp, oysters, and echinoderm systems, a sperm acrosome reaction occurs in close association with the egg and binding results. Although no

FIGURE 20. At approximately 6 hours the first karyokinesis occurs but cytokinesis is suppressed. Bar = 250 μm.

FIGURE 21. Approximately 9 hours post spawning, the second karyokinesis takes place. Bar = 250 μm.

FIGURE 22. After the formation of the four nuclei, first and second cytokinesis occur simultaneously 2–3 hours later and results in a four cell stage. Bar = 250 μm.

acrosome reaction is observed in *M. rosenbergii* sperm, numerous filaments appear to originate from the sperm base. These filamentous elements, possibly pseudopodial in nature, may be involved in a recognition and/or binding phenomenon. These filamentous extensions possibly originate from the vesicular region observed by some authors in the sperm base (Brown, 1967; Koehler, 1979). Additional studies are required, however, to confirm this hypothesis.

Reorientation of the sperm, once initial sperm-egg contact has been established, involves a spike-bending phenomenon. Bending invariably occurs close to the base of the sperm though additional bends are occasionally seen. Movement of the spike in *M. rosenbergii* may either be a passive event (osmotic change) or an active event. A change in osmotic pressure would allow the spike to become less turgid and basically collapse onto the egg surface. Since fertilization is external and in freshwater, a provision for the active movement of either water or ions (activation of channels or pumps) would have to be present to permit the lowering of osmotic (turgor) pressure within the spike. Such a mechanism would suggest a gradual random collapse of the spike. A nondirectional collapse of the spike onto the egg surface does not appear to occur, however. The spike consistently bends at the base, with a secondary bending of the spike occurring as the base is inverting over the breach in the investment.

As an alternative, an active contractile process is suggested by several lines of evidence. Numerous parallel 6 nm filaments, resembling microfilaments, are observed in the spike and radial fibrils of *M. rosenbergii* sperm (Lynn and Clark, 1983). In addition, the radial fibrils are associated with centrioles at the base of the spike and are similar in morphology to rootlets observed in the base of flagellated sperm (Kleve and Clark, 1980). Actin has been reported in the spike of Penaeidae shrimp (Brown *et al.*, 1976) and Clark *et al.* (1981a) suggest that this actin may play an active role in drawing the sperm closer to the egg after spike contact has occurred. In *M. rosenbergii* filaments could differentially contract and cause the spike to bend. A second contractile process may also occur when the base of the sperm is inverted over the spike just prior to the incorporation of the sperm into the egg. Preliminary investigations have been unsuccessful, however, in identifying an actin component in the sperm of *M. rosenbergii* (Lynn, unpublished).

An additional feature observed in the process of fertilization of *M. rosenbergii,* but not limited to the species, is the apparent enzymic digestion of a localized opening in the egg investment. Initially, activity is restricted to the spike tip. Enzymic activity associated with the passage of the sperm through extraoocytic layers has been reported in vertebrates (Yanagamachi and Teichman, 1972; Stambaugh, 1978) as well as invertebrates (Levine *et al.*, 1978; Green and Summers, 1980). In other animal sperm, the enzymic activity has been classically associated with the acrosome and its activation during the acrosome reaction (Stambaugh, 1976; Levine, 1978). Although an obvious acrosome reaction is not observed in *M. rosenbergii* sperm, an enzyme may be localized on/in the spike and released or activated upon contact with the egg investment. If a trypsin-like enzyme is involved as in other animal sperm (Levine *et al.*, 1978; Stambaugh, 1978), its presence could easily be assayed by inhibiting fertilization with known protease inhibitors.

In the present paper, two interesting features of gamete interaction in natantians have been demonstrated: first, a two stage orientation-reorientation of the sperm in contact with the egg, including bending of the sperm spike; and second, the penetration of the egg investments possibly involving lytic enzymes. Fertilization in *M. rosenbergii* may provide a new system for investigation of events such as the enzymic digestion of egg investments and types and functions of contractile systems

in gametes. Studies on these phenomena have been initiated and will be reported in a later paper.

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